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Recovery from behavior and developmental effects of chronic oral methylphenidate following an abstinence period

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Abstract

Chronic oral methylphenidate (MP) exposure in rats is associated with numerous developmental and behavioral consequences. The present study investigated the persistence of the effects of chronic oral MP exposure after abstinence from MP use. Male and female rats were exposed to daily orally self-administered water, low dose MP (LD), or high dose (HD) MP for 13 weeks, followed by a 4-week abstinence period. Fluid, food consumption and bodyweights were monitored and animals were tested for locomotor activity, anxiety- and depressive-like symptoms, learning and memory, and social behavior during both the treatment and abstinence phases of the experiment. During treatment, MP attenuated bodyweight regardless of sex, but increased food and fluid consumption in females and males by 20.7% and 30.1%, respectively. MP also increased locomotor activity in both males and females observed as increased distance travelled in an open field (59.1% and 95.9%, respectively) and increased locomotor activity in the home cage over a 24-hour circadian cycle (45.5% and 63.0%). Additionally, MP exerted an anxiolytic effect observed as increased time spent in the open arms of an elevated plus maze (31.1% in HD males, 59.2% in HD females), and an increased latency to immobility in a forced swim test (330% in HD males, 418% in HD females). The effects of MP (bodyweight, consumption, locomotion, anxiolytic, and anti-depressive) were, almost without exception, eliminated during the abstinence period. MP had no impact on learning and memory performance as measured by a T-maze, or social behavior during treatment. These findings suggest that the behavioral consequences of chronic oral MP treatment in our preclinical model are reversible in rats following an abstinence period from use of the drug.

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Keywords

Methylphenidate; Ritalin; Psychostimulant; Attention Deficit Hyperactivity Disorder; Sensitization; Anxiety

1. Introduction

Methylphenidate (MP; Ritalin and Concerta) is one of the most prescribed psychostimulants for the treatment of Attention Deficit Hyperactivity Disorder (Lakhan and Kirchgessner, 2012). According to the International Narcotics Control Board, MP was dispensed at a record 2.4 billion doses in 2013 (U.N., 2014). Recently, non-prescribed use and abuse of MP has become an alarming phenomenon and there are numerous reports of growing illicit use of MP among high school and college students (Cox et al., 2003, Wilens et al., 2008). This increasing illicit MP use creates concern as it may lead to a surge in the occurrence of adverse consequences of chronic MP exposure such as psychosis, myocardial infarction, cardiomyopathy, and even sudden death, as previously reported (Lakhan and Kirchgessner, 2012). More research is needed to better understand the consequences of chronic MP exposure in a non-ADHD population.

Preclinical studies have reported noteworthy morphological, behavioral, physiological, and cognitive consequences of chronic MP exposure in subjects without a prior psychotic history. Specifically, chronic exposure to high MP doses has been associated with reduced fluid intake and body weight, hyperactivity, and attenuated anxiety in male rats (Robison et al., 2017b) as well as significant effects on dopamine signaling (Robison et al., 2017a). More recent data (Uddin et al., 2018) has shown morphological impairment in appendicular bones associated with chronic oral MP exposure, consistent with prior research (Komatsu et al., 2012), as well as adverse cognitive consequences such as increased impulsivity, neuron and astrocyte loss in the hippocampus, and impaired exploratory activity and object recognition memory following chronic MP exposure (Schmitz et al., 2017).

The effects of chronic exposure to psychostimulants such as MP may persist beyond the cessation of the chronic drug exposure period (see Pierce and Kalivas, 1997 for review). Sensitization to the locomotor-enhancing effect of MP following chronic exposure is one of such effects that has been previously reported (Yang et al., 2003). MP sensitization effects still persist in the short term after drug treatment (McDougall et al., 1999) but nothing is known about how long such effects persist. Besides sensitization, the persistence of other behavioral and physiological effects of chronic MP exposure beyond cessation of drug use are unknown. Moreover, MP is prominently prescribed to patients at a young age, but the rate of prevalence for prescription diminishes by approximately 17% per year throughout adulthood (McCarthy et al., 2009). Illicit use in the non-ADHD population is also distressingly common (Cox et al., 2003, Wilens et al., 2008) in adolescents and young adults for recreational use or as a study aid. For the sake of individuals that administer MP chronically but only for a limited developmental period, it is important to elucidate possible residual effects of chronic MP exposure long after cessation of its use.

Both the route of MP administration and dose are critical determinants of the serum and brain MP levels, as well as its pharmacokinetic profile and behavioral and neuropsychological effects, all of which also underlie the clinical relevance of preclinical findings (Kuczenski and Segal, 2005, Dafny and Yang, 2006). Our lab previously designed a paradigm of oral MP administration in adolescent rats that produced clinically relevant MP pharmacokinetics (Thanos et al., 2015). We recently employed this paradigm to unveil a wide range of physiological and behavioral effects of chronic MP administration from early adolescence through early adulthood that corroborated many previously reported effects of MP administration (Robison et al., 2017b). Using this same paradigm, the present study examined if the effects of chronic MP in rats were persistent or reversed following cessation of treatment (4 weeks).

2. Materials and methods

2.1 Animals

Male (n=36) and female (n=54) Sprague-Dawley rats were obtained from Taconic Farms (Germantown, NY) and divided into either water (W), low dose 4/10 mg/kg MP (LD), or high dose 30/60 mg/kg MP (HD) groups (n = 30/group). On arrival (~PND28), rats were single housed in a temperature- and humidity-controlled room on a reverse 12-hour light cycle (lights off 08:00h). Rat chow (Teklad, Indianapolis, IN) was provided *ad libitum*, and water or MP solution was available during the dark period for 8 hours daily between 09:00h and 17:00h. Food intake, fluid intake, and body weight were recorded daily throughout the experiment. Experiments were conducted in conformity with the National Academy of Science's Guide for the Care and Use of Laboratory Animals (NAS and NRC, 1996) and approved by the State University at Buffalo Institutional Animal Care and Use Committee protocols.

2.2 Experimental Design and Drug Consumption Paradigm

On ~PND35, rats were put on limited 8-hour daily access to MP for 13 consecutive weeks (treatment phase), followed by 4 weeks of abstinence from MP (abstinence phase). MP was delivered to rats as methylphenidate hydrochloride (Sigma-Aldrich, St Louis, MO) in water (vehicle) for 8 hours daily during the treatment phase of the experiment as previously described (Thanos et al., 2015). Briefly, rats received either a 4 mg/kg (LD) or 30 mg/kg MP (HD) during the first hour of access, and 10 mg/kg (LD) or 60 mg/kg MP (HD) for the remaining 7 hours of access. We have reported this paradigm to produce peak serum concentrations of 8 ng/ml for the 4/10 mg/kg (LD) group and 30 ng/ml for the 30/60 mg/kg (HD) group.

2.3 Behavioral Tests

2.3.1 Open field locomotor activity—Locomotor activity was assessed weekly during both treatment and abstinence phases for 90 minutes in an open-field chamber as previously described (Thanos et al., 2015). Rats were tested during the dark cycle between 11:00h and 16:30h in an arena (dimensions 40.64 cm × 40.64 cm × 40.64 cm, 2.54 cm beam space and 1.27 cm spatial resolution) that is equipped with a photo beam activity monitoring system (Coulbourn Instruments, Allentown, PA). Open field locomotor activity data was acquired

with TruScan v2.0 software and horizontal distance travelled and velocity, number of entries and time spent in the vertical plane, and both distance and time spent in the center of the chamber were analyzed.

2.3.2 24-Hour Home-Cage Locomotor Activity—24-hour home-cage locomotor activity was assessed as previously described (Robison et al., 2017b) during weeks 1, 2, 4, 8, and 13 of the treatment phase and weeks 1, 2, and 4 of the abstinence phase. Locomotor activity during a full 24-hour circadian cycle was designated by mean total beam breaks, measured using a photo beam activity monitor attached to the cage top of each animal's original home cage (50 cm × 25 cm × 30 cm high) (Starr Life Sciences Corp, VitalView software 1.1; Oakmont, Pennsylvania). Throughout the 24-hour experiment, *ad libitum* access to chow and the 8-hour limited access-drinking paradigm were maintained.

2.3.3 Elevated Plus Maze—Anxiety-like behavior was assessed on an elevated plus maze during treatment week 12 and abstinence weeks 1 and 4 as previously described (Robison et al., 2017b). Rats were tested during the dark cycle between 11:00h and 16:30h. Rats were placed on the center of the elevated plus maze and then allowed to explore the four arms of the maze for 5 minutes. Anxiety was assessed as the proportion of the total test time that a rat spent in the open arm. Behavior was recorded using D-Link cameras and software (D-Link Corporation Taipei, Taiwan), and analyzed using TopScan behavior image analysis software (Clever Sys Inc Reston, VA).

2.3.4 Forced Swim Test—Forced swim behavior was assessed in all rats during week 12 of treatment and during week 1 of abstinence using the forced swim test (FST), which is an established and well-validated measure of depressive-like behavior in rats (Walf and Frye, 2007). Buckets measuring 39cm × 27cm (height × diameter) were filled with room temperature water and rats were given a 15-minute habituation session in the apparatus 24 hours prior to the testing session, which lasted for 5 minutes. Testing was conducted between 11:00h and 16:30h, and recorded with D-Link cameras and software (D-Link Corporation Taipei, Taiwan), then analyzed with TopScan software (Clever Sys Inc., Reston, Virginia). Behaviors measured during the 5-minute testing period were climbing, swimming and latency to immobility.

2.3.5 Social Interaction—Interest in social interaction was assessed during week 12 of chronic MP treatment and during weeks 1 and 4 of abstinence as previously described (Robison et al., 2017b). In summary, testing was performed in a room illuminated only by red light during the dark cycle between 11:00h and 17:00h. The test was performed in a social interaction arena consisting of a square unit with three chambers divided by partition, each measuring 20cm by 60 cm. Identical metal cages measuring 21.59cm × 12.7cm × 11.43cm (length × width × height) were located in diametrically opposed corners of the left and right chambers. During a 3-minute habituation session that preceded testing, the test rat was placed in the center of the arena alone and allowed to explore all chambers. During the test session that commenced 30 minutes after the habituation ended, a novel rat was placed under one of the square cages after which, the test rat was placed in the center of the arena and allowed to freely explore the arena for five minutes; novel social stimuli were other rats

from the experiment. Test sessions were recorded using D-Link cameras and software (D-Link Corporation Taipei, Taiwan) and the time spent interacting with both cages was rated using the TopScan software (Clever Sys Inc., Reston, Virginia). The social preference index during the test was calculated as a ratio of interaction time with the social cage to the interaction time with both cages. Testing chambers were cleaned with a 10% ammonia solution between runs to eliminate possible confounds from odor cues.

2.3.6 Novel Object Recognition—Short term working memory was assessed using the Novel Object Recognition test as previously described (Moy et al., 2004) and by our lab (Robison et al., 2017b) in week 12 of treatment and weeks 1 and 4 of abstinence. Briefly, animals were placed in the center of an open-field arena of dimensions 40.64 cm × 40.64 cm × 40.64 cm (Coulbourn Instruments, Allentown, PA) with two identical objects (ceramic bud vases) in opposing corners 6cm from the walls of the chamber for a 5-minute acquisition run. After 30 minutes, animals were returned to the arena for a five-minute test run, with one of the two objects replaced by a new object (plastic pink and white striped bucket). The primary dependent variable used was the discrimination index (DI) between the novel and familiar objects, which was calculated as the proportion of time spent exploring the novel object (relative total exploration time) less the proportion of time spent exploring the familiar object (relative to total exploration time). Chambers were cleaned with 10% ammonia between runs to combat possible odor confounds.

2.4 Statistical Analysis

For the treatment phase, analysis of body weight, food intake, fluid intake, open field locomotor activity, and circadian activity were analyzed using independent subjects three-way repeated measures ANOVAs [between-subject factors: drug (MP dose) and sex; within-subjects factor: time (week of treatment)] using SPSS 24.0 statistical software (IBM) with normality and homogeneity of variance assumed. The Greenhouse-Geyer and Huynh Feldt were conducted for sphericity. Two-way repeated measures ANOVAs were subsequently conducted separately within sex [between subjects: drug; within subjects: time] using SigmaPlot 11.0 statistical software. A two-way ANOVA [between-subject factors: drug and sex] was conducted using SigmaPlot to analyze novel object recognition, social interaction, elevated plus maze and forced swim test during treatment. *Post-hoc* analyses of all possible between treatment groups effects were conducted, when justified by an effect of treatment in the ANOVA using Tukey's method. For the abstinence phase, three-way repeated-measures ANOVAs [between-subject factors: drug (MP dose) and sex; within-subject factor: time (week of treatment)] were conducted using SPSS 24.0 statistical software (IBM) to analyze food and fluid consumption, bodyweight, open field locomotor activity, and circadian activity. This model was also used for the novel object recognition, elevated plus maze and forced swim tests as well, as they were each run twice during abstinence. Social interaction was tested using a two-way ANOVA [between-subject factors: drug (MP dose) and sex] using SigmaPlot. Two-way ANOVAs within sex [between-subject factor: drug (MP dose); within-subject factor: time (week of treatment)] as justified by the overall model were run for all other behavioral tests. *Post-hoc* analyses of all possible between treatment groups contrasts were conducted as justified using Tukey's method.

3. Results

3.1 Fluid and food consumption.

3.1.1 Fluid consumption.—Total daily fluid intake was averaged weekly for each treatment group. A three-way repeated measures ANOVA of fluid consumption during the treatment phase revealed main effects of sex [$F_{(1, 82)} = 22.99, p < .001$] and time [$F_{(13, 70)} = 65.37, p < .001$] but no main effect of treatment. The interactions of sex $\times$ time [$F_{(13, 70)} = 13.11, p < .001$], treatment $\times$ time [$F_{(26, 140)} = 3.33, p < .001$], and treatment $\times$ sex [$F_{(2, 82)} = 4.09, p < .05$] were significant. Furthermore, the interaction of treatment $\times$ sex $\times$ time was significant [$F_{(26, 140)} = 1.95, p < .001$]. Females consumed more fluid than males (Figure 1, Table 1).

A separate three-way repeated measures ANOVA of fluid consumption during the abstinence phase revealed main effects of treatment [$F_{(2, 82)} = 16.47, p < .001$], sex [$F_{(1, 82)} = 31.30, p < .001$], and time [$F_{(13, 70)} = 14.06, p < .001$]. The interactions of sex $\times$ time [$F_{(13, 70)} = 5.30, p < .01$], and treatment $\times$ time [$F_{(26, 140)} = 2.36, p < .05$] were also significant, while treatment $\times$ sex [$F_{(2, 82)} = 3.07, p = .052$] only approached significance. Post hoc analyses revealed that regardless of sex, the HD group drank more fluid than both LD and W groups while female rats drank more fluid than male rats (Figure 1, Table 2).

3.1.2 Food Consumption.—Daily food intake was averaged weekly for each treatment group during both treatment and abstinence phases. A three-way repeated measures ANOVA of weekly food consumption during the treatment phase revealed significant main effects of sex [$F_{(1, 82)} = 1134.05, p < .001$] and time [$F_{(13, 70)} = 129.05, p < .001$], but not treatment. The interactions of sex $\times$ time [$F_{(13, 70)} = 24.45, p < .001$], treatment $\times$ time [$F_{(26, 140)} = 12.46, p < .001$], and treatment $\times$ sex [$F_{(2, 82)} = 12.27, p < .001$] were significant. Furthermore, the interaction of treatment $\times$ sex $\times$ time was significant [$F_{(26, 140)} = 2.72, p < .001$] (Figure 2, Table 1).

A separate three-way repeated measures ANOVA of food consumed during the abstinence phase revealed main effects of treatment [$F_{(2, 82)} = 6.94, p < .01$], sex [$F_{(1, 82)} = 250.05, p < .001$], and time [$F_{(13, 70)} = 28.25, p < .001$]. The interactions of sex $\times$ time [$F_{(13, 70)} = 21.05, p < .001$], treatment $\times$ time [$F_{(26, 140)} = 12.56, p < .001$], and treatment $\times$ sex [$F_{(2, 82)} = 16.58, p < .001$] were significant. The three-way interaction of treatment $\times$ sex $\times$ time was significant [$F_{(26, 140)} = 3.55, p < .01$]. Post-hoc analyses revealed that the HD group consumed more food than both LD and W groups and males consumed more food than females (Figure 2, Table 2).

3.1.3 Body weight.—A three-way repeated measures ANOVA of weekly body weight during the treatment phase showed main effects of sex [$F_{(1, 82)} = 981.44, p < .001$] and time [$F_{(13, 70)} = 524.95, p < .001$], while the main effect of treatment [$F_{(2, 82)} = 2.86, p = .062$] approached but failed to reach significance. The interactions of sex $\times$ time [$F_{(13, 70)} = 65.848, p < .001$], treatment $\times$ time [$F_{(26, 140)} = 2.92, p < .001$], and treatment $\times$ sex [$F_{(2, 82)} = 7.74, p < .001$] were significant. Further, the interaction of treatment $\times$ sex $\times$ time was significant [$F_{(26, 140)} = 4.53, p < .001$]. Male rats weighed more than female rats and post

hoc analyses revealed that the HD group weighed less than the LD and water groups (Figure 3, Table 1).

A separate three-way repeated measures ANOVA conducted on data from the abstinence phase showed main effects of sex [$F_{(1, 82)} = 2778.43, p < .001$] and time [$F_{(13, 70)} = 182.90, p < .001$], but not treatment. The interactions of sex $\times$ time [$F_{(13, 70)} = 30.17, p < .001$], treatment $\times$ time [$F_{(26, 140)} = 15.56, p < .001$], and treatment $\times$ sex [$F_{(2, 82)} = 8.27, p < .001$] were significant. The three-way interaction of treatment $\times$ sex $\times$ time [$F_{(26, 140)} = 3.78, p < .01$] was also significant. Post hoc analyses revealed that the W group males weighed more than the HD males all through abstinence phase (Figure 3, Table 2).

3.2 Open Field

3.2.1 Distance travelled.—A three-way repeated measures ANOVA of mean distance travelled revealed main effects of treatment [$F_{(2, 82)} = 39.91, p < .001$] and time [$F_{(13, 70)} = 7.26, p < .001$] but not sex (Figure 4A and Table 1). The interactions of sex $\times$ time [$F_{(13, 70)} = 8.65, p < .001$] and treatment $\times$ time [$F_{(26, 140)} = 9.47, p < .001$] were significant, but not treatment $\times$ sex. The three-way interaction of treatment $\times$ sex $\times$ time was also significant [$F_{(26, 140)} = 2.89, p < .001$].

A separate three-way repeated measures ANOVA conducted on data from the abstinence phase showed a main effect of sex [$F_{(1, 82)} = 43.97, p < .001$] but not treatment or time. The interactions of sex $\times$ time [$F_{(13, 70)} = 8.73, p < .001$] and treatment $\times$ time [$F_{(26, 140)} = 3.05, p < .001$] were found to be significant, while treatment $\times$ sex [$F_{(2, 82)} = 3.07, p = .053$] was found to only approach significance. The three-way interaction was not significant (Figure 4A, Table 2). Data on other measures of locomotor movement including velocity in the open field, rearing events, and time spent rearing were obtained and analyzed as well. Results within both the treatment and abstinence phases were highly consistent with those reported here for distance travelled (results of statistical analysis and relevant figures are available for velocity, rearing events, and rearing time in the open field in Supplemental Figures 1A and 1B, Figures 2A and 2B, and Figures 3A and 3B, respectively, as well as Supplemental Tables 1 and 2).

3.2.2 Center activity.—A three-way repeated measures ANOVA of distance travelled in the center during the treatment phase showed main effects of treatment [$F_{(2, 82)} = 22.98, p < .001$], sex [$F_{(1, 82)} = 88.67, p < .001$], and time [$F_{(13, 70)} = 47.57, p < .001$]. The interactions of sex $\times$ time [$F_{(13, 70)} = 8.56, p < .001$] and treatment $\times$ time [$F_{(26, 140)} = 7.28, p < .001$] were significant, but the treatment $\times$ sex interaction was not. Further, the three-way interaction between treatment $\times$ sex $\times$ time [$F_{(26, 140)} = 1.81, p < .05$] was significant (Figure 4B, Table 1).

A separate three-way repeated measures ANOVA of distance travelled in the center during the abstinence phase showed a main effect of sex [$F_{(1, 82)} = 142.12, p < .001$], but not of treatment or time. The interaction of sex $\times$ time [$F_{(13, 70)} = 11.90, p < .001$] and treatment $\times$ time [$F_{(26, 140)} = 3.55, p < .01$] were significant, but the interaction of treatment $\times$ sex was not. The three-way interaction was not significant (Figure 4B, Table 2).

A three-way repeated measures ANOVA of total time spent in the center of the open field chamber during the treatment phase showed main effects of treatment [$F_{(2, 82)} = 4.56, p < .001$], sex [$F_{(1, 82)} = 17.79, p < .001$], and time [$F_{(13, 70)} = 69.25, p < .001$]. The interaction of sex $\times$ time [$F_{(13, 70)} = 4.95, p < .001$] was significant, but not the treatment $\times$ time or the treatment $\times$ sex interactions. The three-way interaction of treatment $\times$ sex $\times$ time [$F_{(26, 140)} = 1.57, p = .057$] approached but did not reach significance (Figure 4C, Table 1).

A separate three-way repeated measures ANOVA conducted on data from the abstinence phase showed a main effect of sex [$F_{(1, 82)} = 29.19, p < .001$] and time [$F_{(13, 70)} = 5.13, p < .01$] but not of treatment. The interaction of sex $\times$ time [$F_{(13, 70)} = 4.43, p < .01$] was significant, but the interactions of treatment $\times$ time, and treatment $\times$ sex were not. The three-way interaction was not significant.

3.3 Home-Cage 24 Hour Circadian Activity

Home-cage activity, measured as total beam breaks over a 24-hour circadian cycle, was assessed during weeks 1, 2, 4, 8, and 13 of treatment the treatment phase, and weeks 1 and 4 of abstinence. A three-way repeated measures ANOVA of 24-hour home-cage activity during the treatment phase showed main effects of treatment [$F_{(2, 82)} = 20.29, p < .001$], sex [$F_{(1, 82)} = 163.92, p < .001$] and time [$F_{(13, 70)} = 9.09, p < .001$]. The interactions of sex $\times$ time [$F_{(13, 70)} = 5.68, p < .001$], treatment $\times$ time [$F_{(26, 140)} = 3.28, p < .001$], and treatment $\times$ sex [$F_{(2, 82)} = 4.82, p < .05$] were significant. Further, the three-way interaction of treatment $\times$ sex $\times$ time [$F_{(26, 140)} = 3.011, p < .01$] was significant (Figure 5, Table 1).

A separate three-way repeated measures ANOVA conducted on data from the abstinence period showed a significant main effect of sex [$F_{(1, 66)} = 51.983, p < .001$] with greater activity in females than males, but the main effects of time and treatment were not significant. The time $\times$ treatment interaction was significant [$F_{(2, 66)} = 5.194, p < .01$] but the interaction of sex $\times$ time, sex $\times$ group, and the three-way interaction were not significant. Post hoc analyses revealed no between-group difference in either males or females (Figure 5, Table 2). Further analysis was conducted on the active/dark and inactive/light cycles separately. Patterns of activity during the active/dark cycle match those seen in total activity, and no notable effects were observed during the inactive/light cycle (see Supplemental Figures 4A and 4B, and Supplemental Figures 5A and 5B respectively, as well as Supplemental Tables 1 and 2).

3.4 Elevated Plus Maze

Time in the open arms of the elevated plus maze was calculated as a proportion of total time spent in all arms of the elevated plus maze, which was tested during week 13 of treatment and weeks 1 and 4 of abstinence (Figure 6). A two-way ANOVA during the treatment phase showed main effects of treatment [$F_{(2, 84)} = 4.103, p < .05$] but not sex, nor their interaction. High dose rats spent more time in the open arm than LD rats, with no differences observed between W group treated rats and either HD or LD groups. Post hoc analyses revealed that the HD females spent more time in the open arms compared to water treated females ($p < .05$) with a tendency toward greater time in the open arms compared to the LD females ($p = .$

073). In males, there was also a tendency for greater time spent in the open arm by the HD group compared to the LD group ($p = .052$).

A separate three-way repeated measures ANOVA conducted on the abstinence sessions showed no main effect of treatment, sex or time, but a significant interaction of sex and time [$F_{(2, 81)} = 4.67, p < .05$] while no other interactions were significant. Subsequent ANOVAs within sex for each of the two abstinence weeks showed no main effect of treatment for either sex during either of the abstinence weeks (Figure 6).

3.5 Forced Swim Test.

The forced swim test was conducted during week 12 of treatment and week 4 of abstinence (Figure 7). Two-way ANOVAs were performed for each of the two test sessions separately. During treatment, there was a significant main effect of treatment [$F_{(2, 82)} = 14.83, p < .001$] but not of sex, nor of their interaction. During abstinence, a significant main effect of sex [$F_{(1, 82)} = 20.54, p < .001$] was observed, but not of treatment, nor of their interaction. The HD treated rats had longer latency to immobility than both LD and water treated animals among both males and females. No effect was not found during abstinence (Figure 7).

3.6 Social Interaction.

The social interaction test was conducted during week 13 of treatment and weeks 1 and 4 of abstinence. A two-way ANOVA was performed on the session conducted during the treatment phase, but no significant effects of treatment, sex, or their interaction were found. A three-way repeated measures ANOVA during the abstinence sessions showed significant main effects of sex [$F_{(1, 82)} = 82.41, p < .001$] and time [$F_{(13, 70)} = 14.43, p < .001$], but not treatment. The interaction of sex $\times$ time [$F_{(13, 70)} = 14.60, p < .001$] was significant but the interactions of treatment $\times$ time and treatment $\times$ sex were not. The three-way interaction was not significant.

3.7 Novel Object Recognition.

The novel object recognition test was conducted during week 12 of treatment and weeks 1 and 4 of abstinence. A two-way ANOVA was performed on the session conducted during the treatment phase, but no significant effects of treatment, sex, or their interaction were found. A three-way repeated measures ANOVA was conducted on the abstinence sessions showed significant main effects of sex [$F_{(1, 82)} = 5.13, p < .05$], but not treatment or time. No interactions were significant.

4. Discussion

This study examined the reversibility of the behavioral and developmental effects of chronic MP exposure as were previously reported (Robison et al 2017). We hypothesized that following a period of abstinence from MP, the behavioral consequences of chronic MP exposure will be reversed. Consistent with our hypothesis, we found evidence in several behavioral tests of the reversibility of the behavioral effects associated with chronic oral MP administration. These findings are important, given the substantial number of healthy

adolescents taking MP illicitly (Olfson et al., 2002, Wilens et al., 2008) but subsequently after discontinuing treatment (Cox et al., 2003, Marcus et al., 2005, McCarthy et al., 2009).

In the present study, chronic MP treatment was associated with sex-dependent differences in fluid and food intake as previous studies similarly reported (Goldfield et al., 2007, Goldfield et al., 2011, Davis et al., 2012). Although previous studies collectively agree that chronic MP treatment modulates fluid and food intake, their findings have been inconsistent. While some studies reported a reduction (Goldfield et al., 2007, Elfers and Roth, 2011, Goldfield et al., 2011, Robison et al., 2017b), others have reported an increase (Alam and Najam, 2015, 2017). These inconsistencies across studies may be due to differences in methodology such as the dose administered, route and duration of administration, and strain or species employed as these factors reportedly modulate the effects of chronic MP treatment (Gal et al., 1977, Aoyama et al., 1997, Gerasimov et al., 2000, Yang et al., 2003). These findings corroborate our previous report of decreased fluid consumption in the HD male groups while the female HD group consumed significantly more fluid (Robison et al., 2017b). Further agreeing with our previous report (Robison et al., 2017b), chronic MP had no significant effect on food intake in males, while the HD females consumed significantly more food compared to LD and W groups.

Prolonged abstinence from MP consumption was expected to eliminate the group differences in food and fluid intake following chronic MP consumption, leading to recovery. Interestingly, fluid consumption became significantly higher in the male HD group during the abstinence period compared to their LD and water counterparts while the high fluid and food intake in female HD groups during the treatment phase persisted through the abstinence phase. However, our observation of females suggests that chronic MP causes changes in food and fluid consumption that may be sex-dependent and irreversible despite prolonged MP abstinence.

Regardless of sex, we observed significantly lower bodyweight in the HD group compared to both water and LD groups, which emerged during the first week of treatment and persisted throughout the rest of the weeks. This is consistent with our previous report (Robison et al., 2017b) and corroborates findings from previous studies of chronic MP-induced growth inhibition (Elfers and Roth, 2011, Alam and Najam, 2015, 2017). Collectively, our results and previous reports agree that chronic MP treatment attenuates the growth rate with particular emphasis on weight loss. Surprisingly, there was a disconnect between food consumption and weight gain in the present study as HD-treated females that consumed more food weighed less and HD males weighed less despite similar food consumption compared to both water and LD-treated males. This disconnect between food intake and body weight suggests that additional physiological mechanisms may be responsible for the lower weight associated with HD MP treatment. Indeed, previous studies reported that chronic MP treatment significantly increased energy metabolism within the brain (Comim et al., 2014) and energy expenditure in healthy adult humans (Lorello et al., 2008). Based on these findings, it is plausible that the decreased weight in our male and female HD-treated groups is indicative of an increase in energy metabolism that is characteristic of MP. Alternatively, our findings of greater activity in the open field, as well as on the circadian activity test, support an explanation for the discrepancy between food

consumption and weight driven by greater locomotion generally and thus higher energy expenditure.

In the present study, chronic MP treatment was associated with greater distance traveled, velocity, rearing behavior, and time spent in the center of an open field. This observation corroborates previous reports of MP-induced increase in exploration in an open field and greater general locomotor activity from our lab (Thanos et al., 2015, Robison et al., 2017b) and others (Kuczenski and Segal, 2001, Yang et al., 2003, Dafny and Yang, 2006, Chelaru et al., 2012). Our results from monitoring 24-hour circadian activity also corroborate previous reports (Tirosh et al., 1993, Kent et al., 1995, Thanos et al., 2015, Robison et al., 2017b) and further attest to the MP-associated increase in locomotor activity as we observed in the open field. Our findings of MP-induced increases in locomotor activity in both LD- and HD-treated females but only HD- treated males further supports previous reports of greater sensitivity to psychostimulants in females compared to males (Dafny and Yang, 2006, Robison et al., 2017b).

Two major findings from the present study suggest that the increase in locomotor activity may be elicited only in the physiological presence of MP. First, the reversal of MP-related increase in locomotor activity in the open field during the abstinence period when compared to the treatment period. A similar observation was reported by Antle and colleagues (2012) in mice showing reversal in MP-induced locomotor activity following a one-week abstinence period (Antle et al., 2012). Second, we note the presence of MP-induced increases in locomotion during the dark cycle phase of circadian testing, during the hours when the subjects consumed MP, but not during the light cycle phase when they were deprived. This corroborates a previous observation by (Gaytan et al., 1996) that MP-associated increases in locomotor activity were absent following 24 hours of MP abstinence. These findings, together with previous reports, indicate that MP-induced increases in locomotor activity are reversible following abstinence.

Our findings also reveal a sex-dependent anxiolytic effect of MP as demonstrated by a greater proportion of time spent in the open arms of an elevated plus maze in the female HD group compared to the female water group. This finding is concordant with previous reports of MP-induced anxiolytic effects both from our team (Thanos et al., 2015, Robison et al., 2017b), and others (Barrickman et al., 1995, Bouffard et al., 2003, Zhu et al., 2010). As expected during the abstinence phase, we found no significant differences in anxiety-like behaviors between the treatment and control groups, again demonstrating the recoverability of the effect of MP.

An anti-depressant-like effect of MP was seen in increased latency to immobility in the forced swim test in the HD groups compared to the water groups in both sexes, and additionally compared to the LD group in males. Although our findings are consistent with MP's reported efficacy in humans to treat depression-like symptoms (Hardy, 2009), they contradict other reports of a depressive-like effect of MP (Bolanos et al., 2003, Carlezon et al., 2003, Brookshire and Jones, 2012) in rodents. This may be due to other rodent studies' shorter period of chronic MP administration, or differences in administration routes compared to our oral dosing paradigm, which more closely mimics the pharmacokinetic

profile of slow release tablets in humans (Thanos et al., 2015). Following cessation of MP use during the abstinence period, the antidepressant-like effect of MP was not observed, suggesting that this effect is only elicited in the physiological presence of the drug.

MP had also no significant effect on social interaction regardless of sex or dose during both the treatment and abstinence phases. Since we compared the time a subject spent investigating a cage containing another versus an empty cage rather than the social play behaviors, our paradigm assesses social interest rather than social play. Therefore, our finding is consistent with a previous study, which reported that MP had no significant effect on social interest although it abolished social play (Vanderschuren et al., 2008). As with social interaction, MP demonstrated no significant effect on novel object recognition. Previous investigation of MP's impact on cognitive function in preclinical models has produced inconsistent results. While some studies reported that MP disrupted novel object exploration at an acute 5mg/kg dose (Heyser et al., 2004), memory formation at a 10mg/kg acute dose, (Chuhan and Taukulis, 2006), and in complex operant tasks (Mayorga et al., 2000), other studies, especially clinical studies report cognitive improvement following MP administration (Arnsten and Dudley, 2005, Linssen et al., 2014). Possible reasons for inconsistent findings across studies may include variations in doses and duration of MP administration as well as the paradigm employed to assess cognitive functions. Collectively, these findings suggest that further investigation is necessary in order to elucidate the full impact of MP on cognitive function and depressive-like behaviors.

Some limitations of the present study that may have affected our results merit acknowledgement. Subjects were, by necessity, single-housed for this experiment for our dosing paradigm. However, social isolation is an established stressor triggering anxiogenic behavior in an open field and depressive-like behavior in the forced swim test (Djordjevic et al., 2012, Zanier-Gomes et al., 2015, Ieraci et al., 2016), as well as reduced exploratory and social behavior (Varty et al., 2000, Wooters et al., 2011, Gill et al., 2013). Housing conditions may have interacted with MP's effects on the behaviors that were assessed here. Further, subjects were water deprived for 16 hours, which may serve have served as a chronic stressor, influencing the behaviors that we tested (Heiderstadt et al., 2000).

Conclusions

The results from the present study demonstrate clearly that the developmental and behavioral consequences of chronic oral administration of MP are reversible. These findings corroborate our previous work showing chronic MP-induced increases in dopamine transporter and dopamine type-1 receptor were reversed following 1 month of abstinence (Robison et al., 2017a). The reversal of MP-related changes in behavior after as little as one week of abstinence suggests recovery may occur more rapidly than expected. Our findings of elevated home-cage activity only during the hours when MP was available strongly suggest that the behavioral effects of MP are particularly triggered by its physiological presence. Hence, MP self-administration in healthy individuals may lack residual behavioral effects after physiological clearance of the drug.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

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FIGURE 1 | Mean (+SEM) daily fluid intake.

Fluid consumption tended to increase throughout adolescence and into adulthood in all groups. HD male rats drank less than water rats from the start of the experiment through week 7, 9, and 11 ($^*p < .05$); HD male rats also drank less than LD male rats prior to treatment and at weeks 2, 3, 6, and 7 ($^{\$}p < .05$). HD female rats drank more than LD female rats during week 7, and 9–13 ($^{\wedge}p < .05$); water treated female rats drank more than LD female rats for weeks 8, 10, and 12 ($^{\&}p < .05$). During the abstinence period, HD male rats drank more than LD male rats during weeks 1–4 ($^{\$}p < .05$); HD male rats drank more than water treated male rats during week 1–4 ($^*p < .05$). During the abstinence period, HD female rats drank more than LD female rats during weeks 1–4 ($^{\%}p < .05$); HD female rats drank more than water treated female rats during week 1–4 ($^{\wedge}p < .05$). **(Inset) Mean (+SEM) daily fluid intake in the final treatment and abstinence weeks.** Near the end of the treatment phase, during week 12 of treatment, water treated female rats drank more than LD female rats ($^{\&}p < .05$); HD female rats drank more than LD female rats ($^{\wedge}p < .05$). During the final week of abstinence (week 4), HD male rats drank more than LD male rats ($^{\$}p < .05$); HD male rats also drank more than water treated male rats ($^*p < .05$). During the final

week of abstinence, HD female rats drank more than LD female rats ($^{\wedge}p < .05$); HD female rats also drank more than water treated female rats ($^{\%}p < .05$).

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FIGURE 2 | Mean (+SEM) daily food intake.

Food consumption tended to increase throughout early adolescence before generally tapering off in early adulthood. HD male rats ate less than water treated males from the beginning of the experiment through week 6, and during week 8 ($*p < .05$); HD male rats ate more than water treated males, however, in weeks 11 and 13 ($*p < .05$); HD male rats ate less than LD male rats before treatment began and at weeks 3 and 6 ($*p < .05$); HD male rats ate more, however, than LD male rats during week 13 of treatment ($*p < .05$); LD male rats ate more than water treated rats in week 11 ($*p < .05$). HD female rats ate more than LD female rats in week 4, and from weeks 6–13 ($*p < .05$); HD female rats ate more than water treated rats during weeks 5–13 ($*p < .05$); LD female rats ate more than water treated rats during week 13 ($*p < .05$). During the abstinence period, HD female rats ate more than LD female rats during all weeks, 1–4 ($*p < .05$); HD female rats ate more than water treated female rats in weeks 1–3 ($*p < .05$). (Inset) Mean (+SEM) daily food intake in the final treatment and abstinence weeks. Near the end of the treatment phase, during week 12 of treatment, HD female rats ate more than both water treated female rats ($*p < .05$) and LD female rats ($*p < .05$). During the final week of abstinence, HD female rats ate more than water treated female rats ($*p < .05$).

FIGURE 3 | Mean (+SEM) body weight by week.

Body weights were taken daily and averaged across weeks for each group. Body weight increased steadily throughout adolescence and continued on into adulthood. HD male rats weighed less than water treated male rats throughout the treatment phase of testing, during weeks 1–13 ($*p < .05$); HD male rats also weighed less than LD male rats throughout the treatment phase of testing, during weeks 1–13 ($^{\$}p < .05$). HD female rats weighed less than water treated female rats throughout the treatment phase of testing, during weeks 1–13 ($^{\%}p < .05$); HD female rats also weighed less than LD female rats throughout the treatment phase of testing, during weeks 1–13 ($^{\wedge}p < .05$). During the abstinence period, HD male rats weighed less than LD male rats during all weeks, 1–4 ($^{\$}p < .05$); HD male rats weighed less than water treated male rats during all weeks, 1–4 ($*p < .05$). **(Inset) Mean (+SEM) body weight by week in the final treatment and abstinence weeks.** Near the end of the treatment phase, during week 12 of treatment, HD male rats weighed less than water treated male rats ($*p < .05$); HD male rats weighed less than LD male rats ($^{\$}p < .05$); HD female rats weighed less than water treated female rats ($^{\%}p < .05$); HD female rats weighed less than LD female rats ($^{\wedge}p < .05$). During the final week of abstinence, HD male rats weighed less than water treated male rats ($*p < .05$); HD male rats weighed less than LD male rats ($^{\$}p < .05$).

FIGURE 4A | Mean (+SEM) distance travelled in the open field.

Rats moved at a moderately consistent rate throughout the experiment, with some groups having marked increases late in the treatment phase that dissipated during abstinence. HD male rats moved further than water treated male rats during weeks 3 and 5–13 ($*p < .05$); HD male rats also moved further than LD male rats during weeks 5 and 7–13 ($^{\$}p < .05$). HD female rats moved further than water treated female rats during weeks 4–13 ($^{\%}p < .05$); HD female rats moved further than LD female rats during weeks 4–13 ($^{\wedge}p < .05$). During the abstinence period, HD male rats moved further than water treated males in weeks 2 and 3 ($^{\#}p < .05$); LD male rats moved more than water treated male rats during weeks 1 and 2 ($^{\#}p < .05$). (Inset) Mean (+SEM) distance travelled in the open field in the final treatment and abstinence weeks. Near the end of the treatment phase, during week 12 of treatment, HD male rats moved further than water treated male rats ($*p < .05$); HD male rats moved further than LD male rats ($^{\$}p < .05$). HD female rats moved further than water treated female rats ($^{\%}p < .05$); HD female rats moved further than LD female rats ($^{\wedge}p < .05$). During the final week of abstinence, between treatment-group differences in distance moved were not observed within the sexes.

FIGURE 4B | Mean (+SEM) center distance in the open field arena.

Animals tended to gradually increase distance moved in the center of the open field arena through the course of the experiment. HD male rats moved more in the center than water treated male rats during weeks 6–13 ($*p < .05$); HD male rats moved more in the center than LD male rats during weeks 7–9 and 11–13 ($^{\$}p < .05$). HD female rats moved more in the center than water treated female rats during weeks 3–13 ($^{\%}p < .05$); HD female rats moved more in the center than LD female rats during weeks 6–11 and 13 ($^{\wedge}p < .05$); LD female rats moved more in the center than water treated female rats during weeks 2–4, and 8–11 ($^{\&}p < .05$). During the abstinence period, LD male rats moved more in the center than HD male rats during week 4 ($^{\$}p < .05$). HD female rats moved more in the center than LD female rats in week 3 ($^{\wedge}p < .05$); HD female rats moved more in the center than water treated female rats in week 3 ($^{\%}p < .05$). **(Inset) Mean (+SEM) center distance travelled in the open field in the final treatment and abstinence weeks.** Near the end of the treatment phase, during week 12 of treatment, HD male rats moved more in the center than water treated male rats ($^{\wedge}p < .05$); HD male rats also moved more in the center compared to LD male rats ($^{\$}p < .05$). HD female rats moved more in the center than water treated female rats ($^{\%}p < .05$). During the final week of abstinence, HD male rats moved less in the center compared to LD male rats ($^{\$}p < .05$).

FIGURE 4C | Mean (+SEM) center time in the open field arena.

Animals spent more time in the center of the open field arena throughout the duration of the experiment. HD female rats spent more time in the center than water treated female rats during weeks 3, 4, and 6–11 ($^{*}p < .05$); HD female rats spent more time in the center than LD female rats during weeks 7, 9, and 11 ($^{\wedge}p < .05$); LD female rats spent more time in the center than water treated female rats during weeks 2–4 ($^{\&}p < .05$). During the abstinence period, HD female rats moved more in the center than LD female rats in week 3 ($^{\wedge}p < .05$); HD female rats moved more in the center than water treated female rats in week 3 ($^{*}p < .05$). **(Inset) Mean (+SEM) time spent in center in the final treatment and abstinence weeks.** Near the end of the treatment phase, during week 12 of treatment, no between treatment-group differences were seen in male or female rats. During the final week of abstinence, no between treatment-group differences were seen in male or female rats.

FIGURE 5 | Mean (+SEM) total home-cage activity during a 24-hour circadian cycle.

General activity in the 24 hour circadian activity test was generally stable for most of the treatment phase of the experiment before undergoing a slight fall-off during the abstinence phase. HD male rats had a greater number of beam breaks than water treated male rats during weeks 1–13 ($*p < .05$); HD male rats had a greater number of beam breaks than LD male rats during weeks 1, 8, and 13 ($^{\$}p < .05$). HD female rats had a greater number of beam breaks than water treated female rats during weeks 1–13 ($^{\%}p < .05$); HD female rats had a greater number of beam breaks than LD female rats during weeks 2–13 ($^{\wedge}p < .05$); LD female rats had a greater number of beam breaks than water treated female rats during weeks 8 and 13 ($^{\&}p < .05$). During the abstinence period, no between treatment-group differences were found within the sexes. **(Inset) Mean (+SEM) total home-cage activity during a 24-hour circadian cycle by week in the final treatment and abstinence weeks.** Near the end of the treatment phase, during week 13 of treatment, HD male rats had a greater number of beam breaks than water treated male rats ($*p < .05$); HD male rats also had a greater number of beam breaks than LD male rats ($^{\$}p < .05$). HD female rats made more beam breaks than water treated female rats ($^{\%}p < .05$); HD female rats made more beam breaks than LD female rats ($^{\wedge}p < .05$); LD female rats made more beam breaks than water treated female

rats ($p < .05$). During the final week of abstinence, no differences were observed between treatment groups within the sexes.

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FIGURE 6 | Proportion of time spent in open arm of the elevated plus maze.

HD female rats spent a greater proportion of time in the open arm of the elevated plus maze than LD females during the session conducted during the drug treatment phase ($^*p < .05$). No differences were observed between treatment groups within the sexes during either test session conducted during the abstinence period.

FIGURE 7 |. Latency to immobility on the forced swim test.

During the test session conducted during the treatment phase, HD male rats had a longer latency to immobility than water treated male rats ($*p < .05$); HD male rats also had a longer latency to immobility than LD male rats ($^{\$}p < .05$). HD female rats had a longer latency to immobility than water treated female rats ($^{\%}p < .05$). During abstinence, no between treatment-group differences were observed within the sexes.

Table 1

Pairwise comparisons of significant main effects of drug treatment group, sex, and their interactions with time during the treatment phase.

	Drug	Sex	Time x Drug	Time x Drug x Sex (Male)	Time x Drug x Sex (Female)
Fluid intake		Female > Male	Water > HD (3) Water > LD (3, 9, 10, 12) HD > LD (12-13)	Water > HD (Pre-7, 9, 11) LD > HD (Pre, 2, 3, 6, 7)	HD > LD (7, 9-13) Water > LD (8, 10, 12)
Food intake		Male > Female	HD > Water (11, 13) HD > LD (11-13)	Water > HD (Pre-6, 8) HD > Water (11, 13) LD > HD (Pre, 3, 6) HD > LD (13) LD > Water (11)	HD > LD (4, 6-13) HD > Water (5-13) LD > Water (13)
Body weight	HD < LD, Water	Male > Female	Water > HD (8-13) LD > HD (11)	Water > HD (1-13) LD > HD (1-13)	Water > HD (1-13) LD > HD (1-13)
Circadian dark cycle	HD > LD, Water	Female > Male	HD > Water (1-13) HD > LD (2-13)	HD > Water (1-13) HD > LD (1, 8, 13)	HD > Water (1-13) HD > LD (4-13)
Circadian light cycle		Male > Female		Water > HD (13)	
Circadian total activity	HD > LD, Water	Female > Male	HD > Water (1-13) HD > LD (2-13)	HD > Water (1-13) HD > LD (1, 8, 13)	HD > Water (1-13) HD > LD (2-13) LD > Water (8, 13)
OF Distance	HD > LD, Water		HD > Water (2-13) HD > LD (4-13)	HD > Water (3, 5-13) HD > LD (5, 7-13)	HD > Water (4-13) HD > LD (4-13)
OF Velocity	HD > LD, Water	Female > Male	HD > Water (2-13) HD > LD (4-13)	HD > Water (3, 5-13) HD > LD (5, 7-13)	HD > Water (2-13) HD > LD (2, 4-13)
OF Rearing	HD > LD, Water	Female > Male	HD > Water (2-13) HD > LD (7-13) LD > Water (2, 4)	HD > Water (3, 9-13) HD > LD (11, 12)	HD > Water (4, 6-13) HD > LD (4, 6-11, 13) LD > Water (2)
OF Rearing time	HD, LD > Water	Female > Male	HD > Water (9, 11, 13) LD > Water (13)	HD > Water (9, 11-13) HD > LD (11) LD > Water (6)	HD > Water (4, 9, 13) LD > Water (2, 5, 8-10, 12, 13)
OF Center distance	HD > LD > Water	Male > Female	HD > Water (3-13) HD > LD (7-13) LD > Water (4, 6, 9, 10)	HD > Water (6-13) HD > LD (7-9, 11-13)	HD > Water (3-13) HD > LD (6-11, 13) LD > Water (2-4, 8-11)
OF Center time	HD > Water	Male > Female	HD > Water (7, 9, 10) LD > Water (2)		HD > Water (3, 4, 6-11) HD > LD (7, 9, 11) LD > Water (2-4)
Elevated plus maze	HD > LD		N/A	N/A	N/A
Forced swim test	HD > LD, Water		N/A	N/A	N/A
Social interaction			N/A	N/A	N/A
Novel object recognition			N/A	N/A	N/A

Table 2

ABSTINENCE PHASE pairwise comparisons of significant main effects of drug treatment group, sex, and their interactions with time.

	Drug	Sex	Time x Drug	Time x Drug x Sex (Male)	Time x Drug x Sex (Female)
Fluid intake	HD > LD HD > Water	Female > Male	HD > LD (1-4) HD > Water (1-4)	HD > LD (1-4) HD > Water (1-4)	HD > LD (1-4) HD > Water (1-4)
Food intake	HD > LD HD > Water	Male > Female	HD > LD (1, 2) HD > Water (1, 2)		HD > LD (1-4) HD > Water (1-3)
Body weight		Male > Female		Water > HD (1-4) LD > HD (1-4)	
Circadian dark cycle		Female > Male		LD > HD (4)	
Circadian light cycle					LD > HD (4)
Circadian total activity		Female > Male	LD > Water (1)		
OF Distance		Male > Female	LD > Water (2) HD > Water (2)	LD > Water (1, 2) HD > Water (2, 3)	
OF Velocity		Female > Male		LD > Water (1, 2) HD > Water (2, 3)	
OF Rearing		Female > Male			
OF Rearing time		Female > Male	LD > Water (2) HD > Water (3)		LD > Water (2-4) LD > HD (2) HD > Water (3)
OF Center distance		Male > Female		LD > HD (4)	HD > LD (3) HD > Water (3)
OF Center time		Male > Female			HD > LD (3) HD > Water (3)
Elevated plus maze (% time in open arm)					
Forced swim test (latency to immobility)		Male > Female	N/A	N/A	N/A
Social interaction (% in social side)		Female > Male			
Novel object recognition		Male > Female			